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In Vitro and *in Vivo* Study of the Expression Vector Encoding Vascular Endothelial Growth Factor

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Abstract. Vascular endothelial growth factor (VEGF) is an angiogenic cytokine with potential therapeutic applications in human diseases. It is a mitogen primarily for endothelial cells. The transfer of the cDNA encoding VEGF to ischemic tissues, which cannot be revascularized otherwise, represents a novel and promising approach to the treatment of vascular disorders. In this work the VEGF165 cDNA was cloned into the expression vector pSecTag2B. The activity of the construct was studied in cell culture as well as *in vivo*. Western blotting study showed that the cells transfected with the vector secreted significantly higher amounts of VEGF to the culture medium than the non-transfected cells. *In vivo* study revealed an increased number of new vessels in animals injected with vector encoding VEGF as compared with empty plasmid. Also, tumor cells transfected with the VEGF plasmid exhibited extensive vascularization.

Key words: angiogenesis; VEGF; gene therapy.

Introduction

It is well documented that vascular endothelial growth factor (VEGF) and its receptors play the crucial roles the in angiogenesis which occurs during normal embryonic development and in tumor growth and metastasis formation [4, 19]. VEGF was orginally described as a permeability factor that increases microvascular permeability *in vivo* with a potency 50 000 times that of histamine[18]. VEGF is specifically mitogenic for endothelial cells and promotes angiogenesis *in vivo*[11, 23]. It is worth mentioning that hypoxia often up-regulates VEGF expression[12]. Four different isoforms of VEGF (VEGF121, VEGF165, VEGF189, VEGF206) have been reported to be expressed in human endothelial cells. VEGF121 and VEGF165 are secreted in soluble form, whereas the two larger isoforms, VEGF189 and VEGF206, remain to be associated with cells[6, 24]. VEGF165 is the most abundantly expressed splice variant, however[7].

Advances in molecular biology have generated methods that are used to construct expression vectors encoding therapeutic factors, for example VEGF[13, 14]. Available data suggest that gene transfer methods may become a therapeutic alternative for the treatment of vascular disorders such as critical ischemia, or the prevention of restenosis. Transfer of the vector encoding VEGF leads to the development of new vessels and, thus, the collateral circulation in pathologic tissue[1, 2, 5, 7–9, 21, 24, 26].

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The aim of this study was to construct an expression vector encoding vascular endothelial growth factor (VEGF165) and to check whether it can induce angiogenesis when introduced into a mouse.

Materials and Methods

DNA amplification. A fragment of DNA encoding VEGF165 was amplified using cDNA from cells of the human urothelial cell line HCV-29 transfected v-raf[17]. Total RNA was extracted from the cultured cells by the method of CHOMCZYŃSKI and SACCHI[3] using TRIZOL reagent (Gibco, USA). The cDNA was synthesized by reverse transcription from 5 mg of RNA with the Superscript Preamplification System (Gibco, USA) using oligo dT as primers. PCR amplification of cDNA was performed using specific primers for VEGF as follow: a denaturation step at 95°C for 5 min, 35 cycles at 95°C for 1 min, 63°C for 1 min and 72°C for 1 min, followed by an elongation step at 72°C for 10 min. The probe was separated by electrophoresis on 1.5% agarose gel with ethidium bromide (0.5 μg/ml and isolated using DNA Gel-out kit (DNA Gdańsk, Poland).

Construction of pSecHVEGF165 expression vector. A pSec expression plasmid (Invitrogen, Holland) with cytomegalovirus (CMV) promoter and zeocin resistance gene was amplified in the *Escherichia coli* DH5α strain growing on LB broth medium (Sigma, USA) containing ampicilin (100 μg/ml). Plasmid was isolated with Wizard DNA Plasmid Isolation Kit (Promega, USA). DNA purity was confirmed spectrophotometrically and by agarose electrophoresis. The isolated plasmid was then digested with BamHI and EcoRI restriction enzyme (Amersham, UK). Amplified cDNA encoding VEGF165 (termed as insert) was digested with the same restrictase. One μg of DNA was digested for 1 h with 10 U of restriction enzyme at 37°C. Both products were then analyzed on agarose gel. The obtained products were then mixed and ligated for 16 h at 16°C in the presence of 1 U of T4 ligase (Amersham, UK). Then, the ligation mixture was transfered into the competent *E. coli*, which were then seeded on LB agar (Sigma, USA) plates and incubated overnight. The bacterial colonies were cultured in LB broth medium containing ampicillin (100 μg/ml) for 16 h. After incubation, each clone was isolated with the DNA Miniprep Kit (DNA Gdańsk, Poland) and analyzed by restrictases digestion (Fig. 1). Next, experiments were performed *in vitro* and *in vivo*.

Stable transfection of OVP 10 and L1 cells. The human ovarian carcinoma OVP 10 cell line and L1 cell line[21] cells were transfected with plasmid pSec/HVEGF165 by the lipotransfection method. The Lipofectin (Gibco, USA) reagent was used according to the manufacturer's protocol. The zeocin resistance clones of the OVP 10 transfected cells were than tested directly for VEGF synthesis. VEGF was measured in the conditioned medium by the Western blotting method.

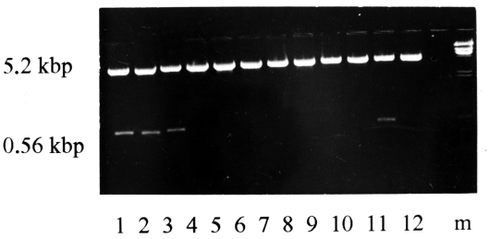

Fig. 1. Restrictase digestion of the pSec/VEGF165 vector from 12 ampicillin resistance clones. The construct was digested with BamHI and EcoRI restriction enzymes; m – marker λ/HindIII; kbp – kilobase pairs

Angiogenesis in vivo assay. Adult inbred female BALB/c mice (9–11 weeks old) received intradermal injections of naked pSec/HVEGF vector or empty construct. The recipients were injected intradermally with 6 doses of 0.1 mg naked vector. A separate group of mice was intradermally injected with 5×10^5 L1 cells transfected with VEGF vector or non-transfected cells. Three, 6 and 13 days later, depending on the experimental conditions, the mice were sacrified and the new blood vessel formation at the inner surface of the skin was counted as described by SIDKY and AUERBACH[19].

Results

Construction of pSec/HVEGF165 expression vector

The human cDNA encoding VEGF165 was cloned into a pSec expression vector. A CMV promoter/enhancer was used to drive VEGF expression and permitted its efficient, high-level expression. The pSec/HVEGF vector also included a Igκ-chain leader signal sequence which allowed the secretion of VEGF, SV40 poliadenylation sequence, zeocin and ampicillin resistance genes. The presence of VEGF insert was confirmed by restrictase digestion (Fig. 1) and sequencing.

The secretion of VEGF to the conditioned medium by the OVP 10 cell stable transfected with pSec/VEGF was studied by Western blotting assay, which showed the presence of VEGF in the transfected clones of the OVP 10 cells. Non-transfected cells did not secrete

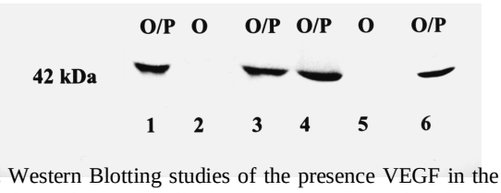

Fig. 2. Western Blotting studies of the presence VEGF in the culture medium of OVP 10 cells. O – OVP 10 non-transfected cells; O/P – OVP 10 cells transfected with vector pSec/VEGF. 1, 2, 3 – 100 μg of total protein per well; 4, 5, 6 – 150 μg of total protein per well

VEGF to the conditioned medium at a level detectible by the method applied (Fig. 2).

In vivo angiogenesis study

The results of the angiogenic *in vivo* assay are shown in Tables 1 and 2. As seen in Table 1, the formation of microvessels after intradermal injection of naked pSec/VEGF vector increased significantly compared with the injection of empty construct. Moreover, plasmid containing VEGF induced long-lasting angiogenesis to 13 days. Table 2 also shows that the L1 cells transfected with vector pSec/VEGF generate a significantly higher number of new vessels than non-transfected cells.

Table 1. The number of blood vessels found in animals intradermally injected with vector pSec/VEGF and empty vector pSec

Day of measurement	VEGF vector	Empty VEGF
3	14.3 ± 3.0	8.6 ± 3.1
	n=30	n=30
6	23.1 ± 7.8	12.5 ± 2.8
	n=13	n=13
13	26.3 ± 4.6	8.7 ± 3.1
	n=14	n=14

Table 2. The number of blood vessels found in animals intradermally injected with L1 cells transfected with vector pSec/VEGF and non-transfected cells

Day of measurement	L1 Cells / VEGF	L1 Cells
3	10.5 ± 11.4	12.6 ± 2.4
	n = 18	n = 18

Discussion

Angiogenesis is defined as the formation of a new capillary network from the pre-existing vasculature[15, 16]. Generally, the turnover of capillary endothelial cells is very slow in physiological conditions. Proliferation of endothelial cells has been observed in wound healing, ischaemia and during tumor progression, however. Many studies have shown the major role of VEGF in this process[4, 11, 23]. Preclinical and clinical findings support VEGF as a promising tool for angiogenic therapy[1, 2, 5, 7–9, 22, 24, 26]. In this study we constructed an expression vector encoding VEGF165 which was tested *in vitro* and *in vivo*. Our results are similar to those obtained in other studies[1, 2, 24, 26] showing that VEGF significantly promotes the development of vessels. In this study, Western blotting assay provided the information that the cells transfected with our vector pSec/VEGF secreted VEGF protein to the culture medium, whereas non-transfected cells did not (Fig. 2). *In vivo* angiogenesis assay revealed an increased number of newly formed vessel in animals injected with naked plasmid pSec/VEGF and cells transfected with pSec/VEGF. In control groups receiving only empty plasmid or non-transfected cells, the number of generated vessels was about 3–4 times lower (Tables 1 and 2). Naked VEGF plasmid when injected intradermally, induced not only a higher number of vessels than empty vector, but also long-lasting angiogenesis. This finding strongly suggests that plasmid was incorporated into the mouse cells, where it survived longer and secreted VEGF. It is worth mentioning that we did not observe any undesirable effects on the experimental animals. Our results are consistent with those of WALDER et al.[27] and proved that VEGF increased vascularity in animals. TAKESHITA et al.[22] and DULAK et al [5] also showed that gene transfer of VEGF165 to the iliac artery of rabbits with induced hindlimb ischemia caused an augmentation of the collateral vessel development. These findings indicated that the plasmids coding angiogenic growth factors could stimulate the development of collateral arteries. Many ensuing studies dealing with angiogenic gene therapy, mainly from ISNER'S group[1, 2, 7, 8, 21, 24, 26], showed that the intramuscular or arterial gene transfer of naked DNA encoding VEGF might have a therapeutic effect. These authors came to the conclusion that ischemic skeletal muscle or arterial miocytes represent good targets for the naked DNA and, therefore, this way of intervention seems to be promising for plasmid-induced gene therapy.

In this study we showed that plasmid coding VEGF significantly promotes vessel development. In our opinion, it may appear to be useful for the treatment of ischemic diseases in the near future. The rationale for this application is based upon studies performed *in vitro* and *in vivo*.

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